Nerve growth factor activates calcium-insensitive protein kinase C-ε in PC-12 rat pheochromocytoma cells

Masahide OHMICHI, Guochang ZHU and Alan R. SALTIEL*

Department of Physiology, University of Michigan School of Medicine, Ann Arbor, MI 48109, and Parke–Davis Pharmaceutical Research Division, Warner–Lambert Company, Ann Arbor, MI 48105, U.S.A.

Protein kinase C (PKC) family members were examined in PC-12 rat pheochromocytoma cells to evaluate their role in the action of nerve growth factor (NGF). Immunoblot analysis of whole cell lysates using antibodies against various PKC isoforms revealed that PC-12 cells contained PKC-α, -δ, -ε and ζ. Assay of the protein kinase activity in these different anti-PKC immunoprecipitates demonstrated that NGF stimulated the kinase activity of PKC-ε, but not PKC-α, -δ and -ζ. Both histone phosphorylation and autophosphorylation of PKC-ε were increased by treatment of PC-12 cells with NGF. This increased phosphorylation observed *in vitro* is rapid, occurring maximally at 2.5 min and declining thereafter. Moreover, this effect of NGF is dose-dependent over physiological concentrations of the growth factor. Although the mechanistic basis for this specificity in PKC activation is not clear, NGF acutely stimulated the production of diacylglycerol without causing corresponding changes in intracellular Ca^{2+} concentrations. These results suggest that NGF may selectively stimulate the Ca^{2+}-insensitive ε isoform of PKC by a phosphatidylinositol-independent mechanism.

INTRODUCTION

Nerve growth factor (NGF) promotes the survival, growth and development of a number of sympathetic and sensory neurons. Although the mechanisms involved in NGF action remain elusive, it is recognized that early changes in protein phosphorylation ultimately play an important role in the cellular actions of the growth factor. A number of serine/threonine kinases have been identified that are activated in response to NGF [1–4]. The best studied of these are a series of enzymes thought to be activated by a linear cascade [5–7]. In this pathway, NGF can activate the $p21^{ras}$ proto-oncogene, followed by the sequential stimulation of *raf*-1 kinase [8], MAP (mitogen-activated protein) kinase kinase [9], MAP kinase [10] and finally ribosomal-protein-S6 kinase II [11]. These serine/threonine phosphorylations are initiated by the direct binding of NGF to its receptor, the phosphoprotein pp140 *trk* proto-oncogene [12–15]. This receptor is a protein tyrosine kinase, and both its activity and tyrosine autophosphorylation are activated in response to NGF.

Although the precise mechanisms whereby the tyrosine kinase activity of the NGF receptor produce the activation of this serine kinase cascade remain unclear, numerous studies [8,16,17] indicate that protein kinase C (PKC) enzymes are not required. Nevertheless, NGF has been reported to activate PKC in PC-12 cells [18,19]. Indeed, phorbol esters reproduce some of the effects of NGF in PC-12 cells [20–23], and PKC inhibitors can block certain actions of NGF [19,24]. NGF stimulates the association of $pp140^{c\text{-}trk}$ with the SH2 domains of phospholipase C (PLC)-γ1, along with the tyrosine phosphorylation of this protein [14,25]. However, despite this effect of the receptor, NGF-induced changes in inositol trisphosphate production or Ca^{2+} mobilization are modest [25] or not observed at all [26]. These findings suggested that PKC activation is mediated by a phosphatidylinositol (PtdIns)-independent pathway [27–29], leading to speculation that different sources of diacylglycerol (DAG) might result in specific activation of isoforms of the enzyme, resulting in a unique spectrum of activities and substrate specificities [30]. We report here that NGF specifically activates the Ca^{2+}-insensitive ε isoform of PKC, as detected in a specific kinase assay *in vitro*, and that this specificity of isoform activation may result from NGF-dependent DAG formation without Ca^{2+} mobilization.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (3000 Ci/mol) was from Du Pont–New England Nuclear (Bannockburn, IL, U.S.A.). ^{125}I-labelled Protein A (30 mCi/mg) was from Amersham (Arlington Heights, IL, U.S.A.). NGF 2.5S was from Bioproducts for Science (Indianapolis, IL, U.S.A.). Receptor-grade epidermal growth factor (EGF) was from Collaborative Research (Lexington, MA, U.S.A.). Insulin was from Eli Lilly (Indianapolis, IN, U.S.A.). Anti-PKC antibodies and phorbol 12-myristate 13-acetate (PMA) were from Gibco–BRL (Grand Island, NY, U.S.A.). PKC substrate (VRKRTLRRL) was from Bachem (Torrance, CA, U.S.A.). Staurosporine, K-252A and H7 were obtained from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were purchased from Sigma and were of the highest quality available.

Immunoblots

PC-12 cells were grown in 60 mm-diam. dishes. Cells were washed once with ice-cold PBS, followed by addition of 100 μl of Laemmli SDS sample buffer [31]. Samples were heated at 100 °C for 5 min, and 30 μg portions of protein were loaded on to SDS/8 %-polyacrylamide gels. Proteins were transferred to nitrocellulose paper and immunoblotted with anti-PKC-α, -β, -γ, -δ, -ε or -ζ antibody as described [32].

Abbreviations used: NGF, nerve growth factor; EGF, epidermal growth factor; PKC, protein kinase C; DAG, diacylglycerol; PtdIns, phosphatidylinositol; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be sent, at: Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI 48105, U.S.A.

Assay *in vitro* of PKC activity in immunoprecipitates

Before hormone treatment, the medium was replaced with serum-free medium and incubated for 1 h. Then 10 nM NGF, 10 nM EGF, 100 nM insulin or 100 nM PMA was directly added to the medium and the incubation was continued for the indicated time at 37 °C. After hormone treatment, the medium was removed and cells were washed once with ice-cold PBS before addition of HNTG lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethanesulphonyl fluoride) [33]. Lysates were cooled to 0 °C for 5 min, followed by addition of 40 μl of rabbit IgG–agarose. After incubation at 4 °C for 15 min, samples were centrifuged at 10000 *g* for 10 min. Anti-PKC-α, -δ, -ϵ, or -ζ antibody was added to the collected supernatants, followed by incubation for 2 h. Immune complexes were then precipitated with Protein A–Sepharose and washed three times with the same lysis buffer, followed by washing with 1 ml of reaction buffer A (20 mM Hepes, pH 7.4, 1 mM $MnCl_2$, 5 mM $MgCl_2$) for the assay of PKC-δ, -ϵ or -ζ, or reaction buffer B (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$) for the assay of PKC-α [34]. Immune precipitates were resuspended in 20 μl of reaction buffer, and 2 μl of 50 μM [γ-^{32}P]ATP (10 μCi) was added, with or without phosphatidylserine and PMA [35]. After incubation for 5 min at 24 °C, reactions were stopped with Laemmli sample buffer, and equal amounts of protein were electrophoresed on SDS/8%-polyacrylamide gels. Immune-complex phosphorylation of histone H1 was assayed in the same manner, except that 1 μg of histone H1 was added to each reaction mixture. For assay of VRKRTLRRL phosphorylation, immunoprecipitates were incubated with 20 μM of this peptide and 50 μM [γ-^{32}P]ATP (3 μCi) in reaction buffer A for 5 min at 24 °C. The reactions were terminated with 15 μl of acetic acid, and spotted on to Whatman p81 paper, followed by washing with 0.75 mM H_3PO_4. Radioactivity was measured by Čerenkov counting. Phosphoamino acid analysis was performed as described previously [36].

Partial purification of PKC from rat brain

Cerebra (approx. 25 g wet wt.) from male Sprague–Dawley rats (200 g) were quickly removed and homogenized in a Teflon–glass homogenizer with 6 vol. of 20 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose, 10 mM EGTA and 2 mM EDTA. The homogenate was centrifuged for 60 min at 100000 *g* and chromatographed on a DE-52 column (14 cm × 3 cm; Whatman) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, 5 mM EGTA and 2 mM EDTA. After washing with the same buffer, the enzyme was eluted with a 1200 ml linear concentration gradient of NaCl (0–0.3 M) at a flow rate of 75 ml/h [37]. PKC activity was assayed in the collected fractions.

Measurements of intracellular Ca^{2+}

The fluorescence of fura-2A-loaded cells was measured by spectrophotometry as previously described [38]. Cells were harvested, pelleted, and resuspended in 10 ml of buffer (30 mM Hepes, pH 7.4, 142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$,6H_2O, 5.6 mM glucose), followed by incubation for 30 min at 37 °C in the presence of 1 mM fura-2A. Fluorescence was measured in a Shimadzu (Kyoto, Japan) spectrophotometer by using the excitation wavelengths 340 and 380 nm and the emission wavelength 510 nm. The fluorescence ratio, displayed as the ordinate, is directly related to intracellular Ca^{2+} concentration [39].

Assay of cellular DAG

PC-12 cells were extracted with chloroform/methanol (1:2, v/v) by a modification of the method of Bligh and Dyer [40]. Samples in the organic phase were dried under N_2, dissolved in 20 μl of 7.5% (w/v) n-octyl β-glucopyranoside/5 mM cardiolipin, and incubated with 0.5 mM [γ-^{32}P]ATP (0.1 μCi) and *Escherichia coli* DAG kinase [41]. The reaction was terminated by addition of 20 μl of 1% (v/v) $HClO_4$ and 450 μl of chloroform/methanol (1:2, v/v), and extracted lipid was quantitatively analysed by silica-gel t.l.c. with the chloroform/methanol/40% methylamine (10:6:1, by vol.) solvent system. The spot corresponding to phosphatidic acid was located by autoradiography and scraped, and radioactivity was measured by Čerenkov counting.

RESULTS

The presence of PKC isoforms in PC-12 cells was evaluated by Western blotting with specific antibodies. Lysates of PC-12 cells were subjected to SDS/PAGE, and immunoblotted with antibodies raised against antigen peptides specific for PKC-α, -β, -γ, -δ, -ϵ and -ζ (Figure 1). All of the antibodies recognized an immunoreactive band from rat brain extracts (lanes 2). However, antibodies against PKC-β and -γ failed to detect immunoreactive proteins in PC-12 cells, indicating that these isoforms are not expressed to significant levels in these cells. In contrast, antibodies against PKC-α, -δ, -ϵ and -ζ detected immunoreactive proteins in PC-12 cells.

To evaluate whether NGF stimulates the specific PKC isoenzymes expressed in PC-12 cells, we analysed their distribution between membrane and cytosolic fractions by immunoblot analysis. Treatment of cells with PMA for 10 min caused the apparent translocation of the α, δ and ϵ isoforms from the cytoplasmic to the membrane fraction, whereas the ζ isoform was unaffected. In contrast, we were unable to detect any

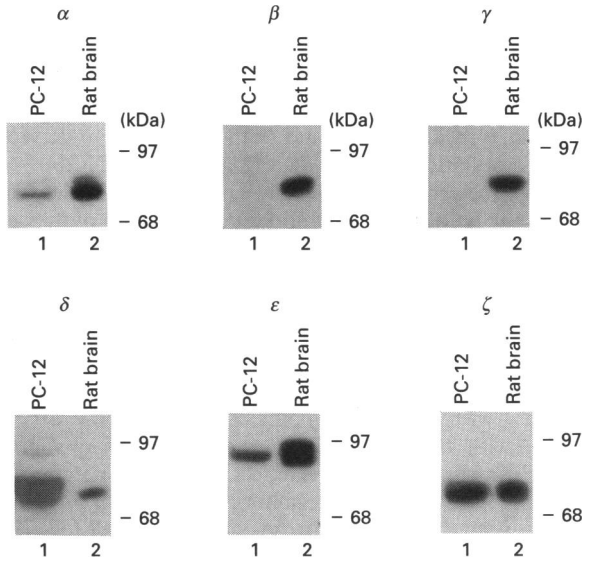

Figure 1 Immunoblot analysis of PKC isoforms in PC-12 cells

Lysates of PC-12 cells (lane 1) or PKC purified from rat brain (lane 2) were prepared as described in the Materials and methods section, and subjected to SDS/PAGE, transferred to nitrocellulose paper,and immunoblotted with antibodies raised against antigen peptides specific for PKC-α, -β, -γ, -δ, -ϵ, or -ζ. For PC-12 lysates, 30 μg of protein was loaded; for rat brain, 20 μg of protein was loaded.

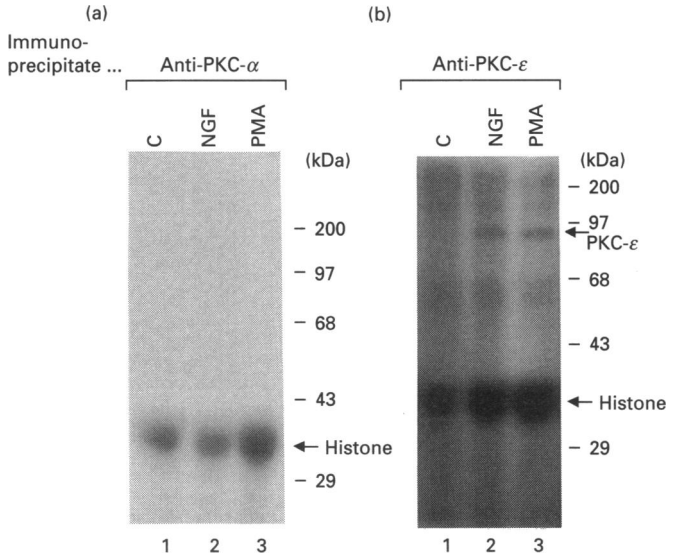

Figure 2 NGF stimulates the activity of PKC-ε in an immunoprecipitate assay

PC-12 cells were grown in 150 mm dishes: 10 nM NGF (lane 2) or 100 nM PMA (lane 3) was directly added to the medium and incubated for 2.5 min at 37 °C (lane 1, C: control). Lysates were immunoprecipitated with anti-PKC-α (**a**) or -ε (**b**) antibody. Immunoprecipitates were incubated with 1 μg of H1 histone and 2 μl of 50 μM [γ-^{32}P]ATP (10 μCi) for 5 min in kinase buffer at 24 °C. Reactions were stopped with Laemmli sample buffer and subjected to SDS/PAGE, followed by autoradiography.

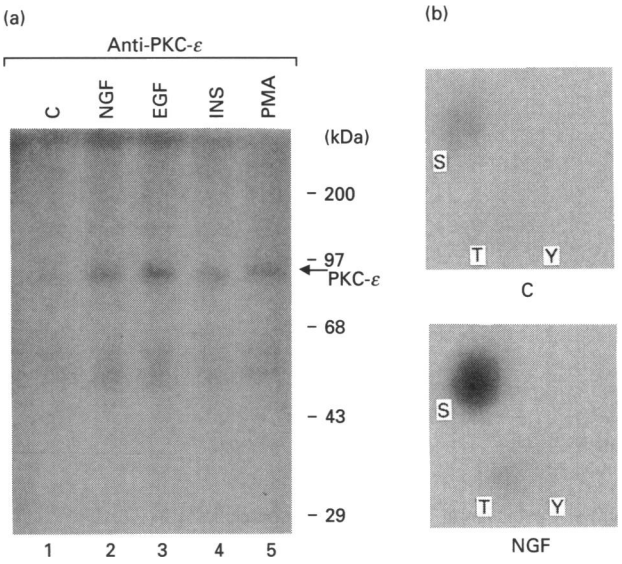

Figure 3 Growth factors stimulate the serine/threonine autophosphorylation of PKC-ε in PC-12 cells

(**a**) PC-12 cells were grown in 150 mm dishes: 10 nM NGF (lane 2), 10 nM EGF (lane 3), 100 nM insulin (INS; lane 4) or 100 nM PMA (lane 5) was directly added to the medium and incubated for 2.5 min at 37 °C (lane 1, C: control). Lysates were incubated with anti-PKC-ε antibody, and immunoprecipitates were incubated with [γ-^{32}P]ATP as described in the legend to Figure 2. Reactions were stopped with Laemmli sample buffer and subjected to SDS/PAGE, followed by autoradiography. (**b**) PKC-ε was phosphorylated *in vitro* in anti-PKC-ε immunoprecipitates from PC-12 cells treated with or without 10 nM NGF for 2.5 min, as described above. The phosphorylated 90 kDa band was excised and subjected to phosphoamino acid analysis by two-dimensional thin-layer electrophoresis. S, T and Y mark the positions of non-radioactive phosphoserine, phosphothreonine and phosphotyrosine respectively.

Table 1 Both NGF and PMA stimulate peptide phosphorylation in anti-PKC-ε immunoprecipitates

PC-12 cells were treated for 2.5 min at 37 °C with or without 10 nM NGF or 100 nM PMA. Lysates were immunoprecipitated with anti-PKC-ε antibody. Immunoprecipitates were incubated with 20 μM VRKRTLRRL peptide and 50 μM [γ-^{32}P]ATP (3 μCi) for 5 min in reaction buffer at 24 °C. The reactions were terminated with 15 μl of acetic acid and phosphopeptides were quantified by filtration.

Treatment	VRKRTLRRL peptide phosphorylation (pmol/min per mg of protein)
Control	736 ± 69
NGF	1301 ± 114
PMA	1209 ± 97

reproducible changes induced by NGF in the distribution of any of these enzymes, despite examination of a range of time points, from 20 s until 15 min (results not shown).

Because of the transient nature of hormone-induced PKC translocation [42], it is possible that we may have missed effects of NGF due to the time limitations of the experiment. Thus we examined the phosphorylation *in vitro* of the different PKC isoforms. PC-12 cells were treated with or without 10 nM NGF or 100 nM PMA for 2.5 min. Cells were lysed and proteins were immunoprecipitated with the specific anti-PKC antibodies, and the resulting immunoprecipitates were subject to a kinase assay *in vitro* with H1 histone and [γ-^{32}P]ATP (Figure 2). Kinase activity could not be detected in anti-PKC-δ or -ζ immunoprecipitates from control cells or cells treated with either agonist (results not shown), suggesting that antisera used in these experiments might block the activity of these isoforms. Treatment of cells with PMA caused increased phosphorylation of H1 histone in the anti-PKC-α immunoprecipitates, although no autophosphorylation of the enzyme was detected (Figure 2a). NGF had no effect on histone phosphorylation or autophosphorylation in anti-PKC-α immunoprecipitates. In contrast, treatment of cells with either PMA or NGF stimulated the phosphorylation of H1 histone in anti-PKC-ε immunoprecipitates (Figures 2b). In addition, both agents increased the autophosphorylation of the 90 kDa PKC-ε. The activation of PKC-ε by both PMA and NGF was verified with the synthetic PKC substrate VRKRTLRRL [18,43]. The phosphorylation of this peptide was stimulated by both NGF and PMA in anti-PKC-ε immunoprecipitates (Table 1).

To explore further the NGF-dependent phosphorylation and activation of PKC-ε, we examined the autophosphorylation *in vitro* in more detail. PC-12 cells were treated with NGF, EGF, insulin or PMA, and lysates were immunoprecipitated with the anti-PKC-ε antibody. The resulting immunoprecipitate was subjected to a kinase assay *in vitro* with [γ-^{32}P]ATP as described above (Figure 3a). NGF, EGF, insulin and PMA were approximately equally effective in stimulating the phosphorylation of PKC-ε in the kinase assay *in vitro*. Moreover, the growth-factor-stimulated phosphorylation of PKC-ε was not dependent on, nor stimulated by, direct addition of phosphatidylserine, Ca^{2+} or PMA to the assay (results not shown). The kinase phosphorylated *in vitro* from the immunoprecipitate assay was subjected to phosphoamino acid analysis (Figure 3b). In cells treated with

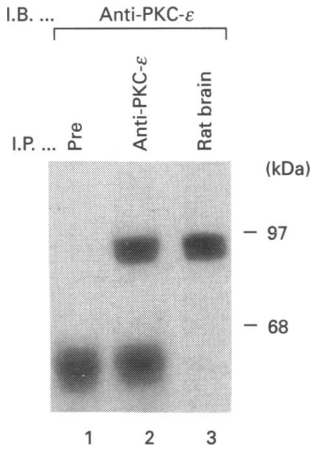

Figure 4 Detection of PKC-ε in immunoprecipitates by Western blotting

PC-12 cells were grown in 150 mm dishes. Lysates were immunoprecipitated (I.P.) with pre-immune rabbit serum (Pre; lane 1) or anti-PKC-ε antibody (lane 2) and subject to SDS/PAGE, followed by immunoblotting (I.B.) with anti-PKC-ε antibody. Lane 3 contains a standard preparation from rat brain.

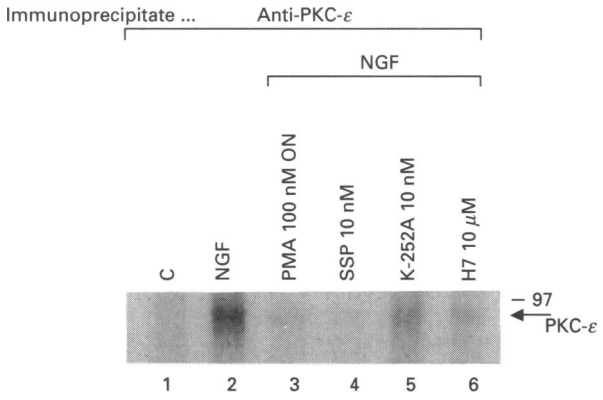

Figure 5 Phosphorylation of PKC-ε induced by NGF is PKC-dependent

PC-12 cells were grown in 150 mm dishes. After preincubation with 100 nM PMA for 16 h (ON) (lane 3) or 10 nM staurosporine (SSP; lane 4), 10 nM K-252A (lane 5), or 10 μM H7 (lane 6) for 10 min, PC-12 cells were treated for 2.5 min with 10 nM NGF (lanes 2–6); lane 1, control (C). Lysates from PC-12 cells were immunoprecipitated with anti-PKC-ε antibody, and immune-complex kinase reactions were performed as described in Figure 3.

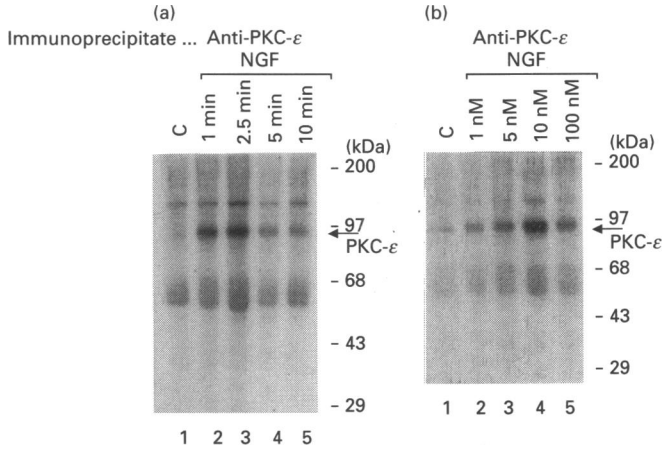

Figure 6 Kinetics of NGF-dependent PKC-ε phosphorylation in PC-12 cells

(**a**) PC-12 cells were treated for the indicated times with 10 nM NGF. After treatment, lysates were immunoprecipitated with anti-PKC-ε antibody, and immune-complex kinase reactions were performed as described in Figure 3. (**b**) PC-12 cells were treated for 2.5 min with the indicated concentration of NGF, and immunoprecipitate kinase reactions were performed as described above. C, control.

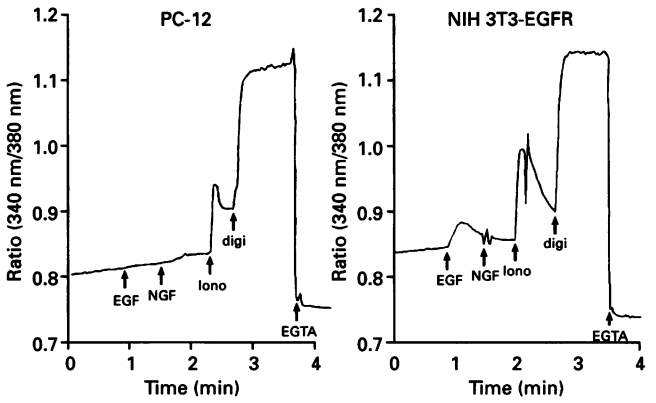

Figure 7 Measurement of intracellular free Ca^{2+} by using fura-2 microfluorimetry

EGF (10 nM), NGF (10 nM), ionomycin (Iono; 10 μM), digitonin (digi; 100 μM) or EGTA (20 mM) was added as indicated to fura-2-loaded PC-12 (**a**) or NIH 3T3-EGFR cells (**b**) at the times indicated by the arrows. Ca^{2+} was measured in a spectrophotometer. The fluorescence ratio, displayed as the ordinate, is directly related to intracellular Ca^{2+} concentration.

NGF, immunoprecipitated PKC-ε contained phosphoserine and a trace of phosphothreonine, with no detectable phosphotyrosine.

To confirm that PKC-ε phosphorylation in the assay *in vitro* was due to autophosphorylation, we confirmed that PKC-ε was present in anti-PKC-ε immunoprecipitates from PC-12 cells by Western blotting with the same antibody (Figure 4). In addition, we examined the effect of PKC inhibitors on the NGF-induced PKC-ε activity. Down-regulation of PKC by incubation with PMA for 16 h, or the addition of staurosporine, K-252A or H7, attenuated the phosphorylation of the 90 kDa PKC-ε protein in the immune-complex assay from cells treated with NGF (Figure 5).

The time course of the stimulation by NGF of PKC-ε autophosphorylation was evaluated (Figure 6a). Phosphorylation of the 90 kDa PKC-ε protein was rapidly increased in immunoprecipitates from cells treated with NGF. Activation of this phosphorylation was observed as early as 1 min, reached a maximum at 2.5 min, and declined thereafter. The concentration-dependence of this effect was also explored (Figure 6b). Treatment of PC-12 cells with as little as 1 nM NGF caused a significant increase in the phosphorylation of PKC-ε. The effect was maximal at 10 nM NGF, but declined slightly at higher concentrations.

Although physiological activation of PKC enzymes generally results from acute increases in cellular DAG content, the mechanistic basis for specific activation of isoenzymes is less clear. In previous studies [30], we could detect no effect of NGF on polyphosphoinositide hydrolysis. To confirm the lack of NGF-dependent PtdIns turnover in these PC-12 cells, we also examined the hormone-induced intracellular mobilization of

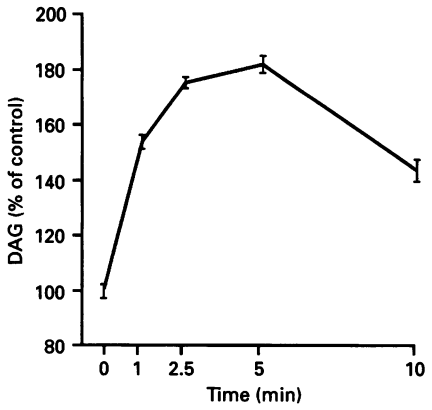

Figure 8 NGF stimulates DAG production

PC-12 cells were grown in 60 mm dishes. At various times after the addition of NGF (10 nM), the DAG was extracted and its mass was determined by phosphorylation to phosphatidic acid with *E. coli* DAG kinase [41]. DAG mass was obtained from the standard curve, and the results (duplicate determinations in two experiments) are expressed as DAG in treated cells as a percentage of that in control PC-12 cells (untreated cells, 33 pmol/mg).

Ca^{2+} (Figure 7). Although both ionomycin and digitonin acutely stimulated the intracellular mobilization of Ca^{2+}, both NGF and EGF had no effect in PC-12 cells (Figure 7a). However, addition of EGF to NIH 3T3 cells over-expressing EGF receptor [44] induced a substantial increase in intracellular Ca^{2+} (Figure 7b).

These results confirmed that PKC-ε activation occurred through a mechanism that did not require PtdIns hydrolysis. We have suggested that NGF-induced DAG may result from the hydrolysis of glycosyl-PtdIns, since the predominantly DAG species that was increased in response to NGF was myristate-labelled [30]. However, the actual mass of DAG produced in these studies was not determined. To evaluate further whether the NGF-stimulated phosphorylation of PKC-ε was due to a significant stimulation of DAG production, the mass of this lipid was assayed by the DAG kinase method [41] in PC-12 cells (Figure 8). NGF treatment caused an acute increase in DAG within 1 min. This effect was maximal at 5 min, with a doubling of DAG mass, and declined thereafter.

DISCUSSION

Members of the PKC family are serine/threonine kinases that were originally discovered by virtue of their Ca^{2+}, phospholipid and DAG dependence. These enzymes are thought to play a key role in the regulation of a variety of cellular processes, including growth, differentiation, secretion and neurotransmitter release [45]. The different PKC isoenzymes are grouped into Ca^{2+}-dependent (PKC-α, -βI, -βII, and -γ) and Ca^{2+}-independent (PKC-δ, -ε and -ζ) enzymes. Although the precise roles of these different family members remain undefined, some preliminary evidence indicates that they may differ with respect to tissue expression [46], substrate specificity [47,48], susceptibility to down-regulation [49,50] and mode of activation [51]. Such results have led several investigators to examine whether some aspects of specificity in signal transduction for certain hormones or growth factors might result from the selective activation of only certain PKC family members [34,52,53]. Similarly to previous observations in primary neurons with insulin [34], we report here that in PC-12 cells NGF selectively activates the ε isoform of PKC, whereas the α isoform is unaffected. Although we were unable to evaluate the effects of NGF on PKC-δ and -ζ, since these activities could not be detected in immunoprecipitated proteins, these data indicate that the Ca^{2+}-dependent isoforms of the enzyme are not activated by the growth factor.

Although the precise mechanism whereby NGF selectively activates PKC-ε remains unknown, it is likely that phosphoinositide hydrolysis is not involved. The growth factor does not exert significant effects on PtdIns turnover or Ca^{2+} mobilization, but does stimulate the hydrolysis of glycosyl-PtdIns in PC-12 cells [30]. NGF-dependent DAG production may also arise via the hydrolysis of other cellular phospholipids, through a phospholipase C or D mechanism [27–29]. In either event, the generation of DAG without the simultaneous mobilization of Ca^{2+} may provide a mode for the selective activation of the Ca^{2+}-insensitive ε isoform of PKC.

The lack of effect of NGF on PtdIns turnover or Ca^{2+} mobilization in PC-12 cells remains puzzling, since the factor markedly stimulates the phosphorylation of PLC-γ1 in these cells. One possible explanation may relate to the magnitude of the effect; fluorescence measurements may not be sufficiently sensitive to detect small changes in Ca^{2+} in these tumour cells. Another possibility is that the binding of $pp140^{c\text{-}trk}$ to PLC-γ1 may play a separate regulatory role, perhaps modulating the binding of other SH2 proteins to the receptor [14]. Conversely, the activity of PLC-γ1 may be subject to cross-regulation, via the NGF-specific 38 kDa associated phosphoprotein [54].

What is the role of PKC-ε activation by NGF? Early reports indicated that NGF-induced neurite-outgrowth could be blocked by the PKC inhibitor staurosporine [55], although later studies [16,56,57] revealed that this compound potently inhibits NGF-receptor tyrosine autophosphorylation. Phorbol esters can mimic some of the effects of NGF, such as neurite outgrowth [58] and c-*fos* induction [59], and lead to phosphorylation of some of the same sites on tyrosine hydroxylase [60] and peripherin [61]. However, down-regulation of PKC did not attenuate NGF-induced differentiation of PC-12 cells [62]. Similarly, the blockade of *ras* isoprenylation did not compromise the effects of NGF on differentiation, phosphorylation of tyrosine hydroxylase or induction of ornithine decarboxylase [63], although other reports indicate that both *ras* and *raf* activation are involved in the stimulation of the MAP kinase pathway and subsequent differentiation of PC-12 cells [5,6]. Taken together, these experiments indicate that pathways involving either $p21^{ras}$ or PKC activation may be sufficient, but not necessary, to mediate some of the effects of NGF, reflecting the redundancy of signalling pathways involved in growth and differentiation.

We thank Dr. Stuart Decker, Dr. Harry LeVine, Dr. David Dudley and Dr. Berta Strulovici for helpful discussions.

REFERENCES

1 Lee, K. Y., Seeley, P. J., Muller, T. H., Helmer-Matyjek, E., Goldstein, M. and Greene, L. A. (1985) Cell **57**, 1101–1107
2 Rawland, E. A., Muller, T. H., Goldstein, M. and Greene, L. A. (1987) J. Biol. Chem. **262**, 7504–7513
3 Vulleit, P. R., Hall, F. L., Mitchell, J. P. and Hardie, D. G. (1989) J. Biol. Chem. **264**, 16292–16298
4 Aletta, T. M., Lewis, S. A., Cowan, N. J. and Green, L. A. (1988) J. Cell Biol. **105**, 1573–1581
5 Wood, K. M., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) Cell **68**, 1041–1050
6 Robbins, D. J., Cheng, M., Zhen, E., Vanderbilt, C. A., Freig, L. A. and Cobb, M. H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6924–6928
7 Hattori, S., Fukuda, M., Yamashita, T., Nakamura, S., Gotoh, Y. and Nishida, E. (1992) J. Biol. Chem. **267**, 20346–20351

8 Ohmichi, M., Pang, L., Decker, S. J. and Saltiel, A. R. (1992) J. Biol. Chem. **267**, 14604–14610
9 Ohmichi, M., Pang, L., Ribon, V. and Saltiel, A. R. (1993) Endocrinology (Baltimore) **133**, 46–56
10 Miyasaka, T., Chao, M. V., Sherline, P. and Saltiel, A. R. (1990) J. Biol. Chem. **265**, 4730–4735
11 Mathuda, Y. and Guroff, G. (1987) J. Biol. Chem. **262**, 2832–2844
12 Kaplan, D. R., Martin-Zanca, D. and Parada, L. F. (1991) Nature (London) **350**, 158–160
13 Klein, R., Jing, S., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991) Cell **65**, 189–197
14 Ohmichi, M., Decker, S. J., Pang, L. and Saltiel, A. R. (1991) Biochem. Biophys. Res. Commun. **179**, 217–233
15 Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V. and Parada, L. F. (1991) Science **252**, 554–557
16 Ohmichi, M., Decker, S. J., Pang, L. and Saltiel, A. R. (1992) Biochemistry **31**, 4034–4039
17 Nakafuku, M., Satoh, T. and Kaziro, Y. (1992) J. Biol. Chem. **267**, 19448–19454
18 Heasley, L. E. and Johnson, G. L. (1989) J. Biol. Chem. **264**, 8646–8652
19 Hama, T., Huang, K. P. and Guroff, G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2353–2357
20 Haycock, J. W., Bennett, W. F., George, R. J. and Waymire, J. C. (1982) J. Biol. Chem. **257**, 13699–13703
21 McTigue, M., Cremins, J. and Haleoguoa, S. (1985) J. Biol. Chem. **260**, 9047–9056
22 Ishii, D., Recio-Pinto, E., Spinelli, W., Mill, J. F. and Sonnenfeld, K. H. (1985) Int. J. Neurosci. **26**, 109–127
23 Greenberg, M., Greene, L. A. and Ziff, E. B. (1985) J. Biol. Chem. **260**, 14101–14111
24 Hall, F. L., Fernyhough, P., Ishii, D. N. and Vulleit, P. R. (1988) J. Biol. Chem. **263**, 4460–4466
25 Kim, U. H., Fink, D., Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G. and Rhee, S. G. (1991) J. Biol. Chem. **266**, 1359–1362
26 Landreth, G., Cohen, P. and Shooter, E. M. (1980) Nature (London) **283**, 202–204
27 Pessin, M. S., Baldassare, J. J. and Raben, D. M. (1990) J. Biol. Chem. **265**, 7959–7966
28 Billah, M. M. and Canthes, J. C. (1990) Biochem. J. **269**, 281–291
29 Brindley, D. N. (1984) Prog. Lipid Res. **23**, 115–133
30 Chan, B. L., Chao, M. V. and Saltiel, A. R. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 1756–1760
31 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
32 Decker, S. J., Ellis, C., Pawson, T. and Velu, T. (1990) J. Biol. Chem. **265**, 7009–7015
33 Margolis, B., Bellot, F., Honegger, A. M., Ullrich, A., Schlessinger, J. and Zilberstein, A. (1990) Mol. Cell. Biol. **10**, 435–441
34 Heidenreich, K. A., Toledo, S. P., Brunton, L. L., Watson, M. J., Daniel-Issakani, S. and Strulovici, B. (1990) J. Biol. Chem. **265**, 15076–15082
35 Bell, R. M., Hannun, Y. and Loomis, C. (1986) Methods Enzymol. **124**, 353–359
36 Decker, S. J. (1984) Mol. Cell. Biol. **4**, 1719–1724
37 Kikkawa, U., Minakuchi, R., Takai, Y. and Nishizuka, Y. (1983) Methods Enzymol. **99**, 288–298
38 Draznin, B., Kao, M. and Sussman, K. E. (1987) Diabetes **36**, 174–178
39 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
40 Bligh, E. A. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
41 Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) J. Biol. Chem. **261**, 8597–8600
42 Kiley, S. C., Parker, P. J., Fabbro, D. and Jaken, S. (1991) J. Biol. Chem. **266**, 23761–23768
43 Davis, R. J. and Czech, M. P. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1974–1978
44 Ohmichi, M., Decker, S. J., Pang, L. and Saltiel, A. R. (1991) J. Biol. Chem. **266**, 14858–14861
45 Nishizuka, Y. (1989) Cancer **63**, 1892–1903
46 Nishizuka, Y. (1988) Nature (London) **334**, 661–665
47 Schaap, D. and Parker, P. J. (1990) J. Biol. Chem. **265**, 7301–7307
48 Marais, R. M., Nguyen, D., Woodget, J. R. and Parker, P. J. (1990) FEBS Lett. **277**, 151–155
49 Pears, C. and Parker, P. J. (1991) FEBS Lett. **284**, 120–122
50 Freisewinkel, I., Riethmacher, D. and Stabel, S. (1991) FEBS Lett. **280**, 262–266
51 Kiley, S., Schaap, D., Parker, P. J., Hsieh, L.-L. and Jaken, S. (1990) J. Biol. Chem. **265**, 15704–15712
52 Kiley, S. C., Parker, P. J., Fabbro, D. and Jaken, S. (1991) J. Biol. Chem. **266**, 23761–23768
53 Pfeffer, L. M., Strulovici, B. and Saltiel, A. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6537–6541
54 Ohmichi, M., Decker, S. J. and Saltiel, A. R. (1992) J. Biol. Chem. **267**, 21601–21606
55 Koizumi, S., Contreras, M. L., Matsuda, Y., Hama, T., Lazarovici, P. and Guroff, G. (1988) J. Neurosci. **8**, 715–721
56 Berg, M. M., Sternberg, D. W., Parada, L. F. and Chao, M. V. (1992) J. Biol. Chem. **267**, 13–16
57 Tapley, P., Lamballe, F. and Barbacid, M. (1992) Oncogene **7**, 371–381
58 Chandler, C. E. and Herschman, H. R. (1980) J. Cell. Physiol. **105**, 275–285
59 Sheng, M., Dougan, S. T., McFadden, G. and Greenberg, M. (1988) Mol. Cell. Biol. **8**, 2787–2796
60 McTigue, M., Cremins, J. and Halegoua, S. (1985) J. Biol. Chem. **260**, 9047–9056
61 Aletta, J. M., Shelanski, M. L. and Greene, L. A. (1989) J. Biol. Chem. **264**, 4619–4627
62 Reinhold, D. S. and Neet, K. E. (1989) J. Biol. Chem. **264**, 3538–3544
63 Qiu, M-S., Pitts, A. F., Winters, T. R. and Green, S. H. (1991) J. Cell Biol. **115**, 795–808

Received 15 April 1993/28 June 1993; accepted 5 July 1993